Distal Interleukin-1 β (IL-1 β) Response Element of Human Matrix Metalloproteinase-13 (MMP-13) Binds Activator Protein 1 (AP-1) Transcription Factors and Regulates Gene Expression*5

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Adam C. Schmucker[‡], Jason B. Wright[§], Michael D. Cole[§], and Constance E. Brinckerhoff^{‡¶}

From the Departments of *Biochemistry, *Genetics, and *Medicine, Dartmouth Medical School, Hanover, New Hampshire 03755

Background: MMP-13 is induced by interleukin-1 β and contributes to cartilage destruction.

Results: We detected activator protein-1, RNA polymerase II, and histone modifications associated with transcription response elements within evolutionarily conserved DNA sequences 20 kb 5' to MMP-13 transcription start site.

Conclusion: The upstream region contains a response element(s) that contributes to MMP-13 gene expression.

Significance: Transcriptional regulation of MMP-13 involves novel interactions among proximal and distal DNA elements.

The collagenase matrix metalloproteinase-13 (MMP-13) plays an important role in the destruction of cartilage in arthritic joints. MMP-13 expression is strongly up-regulated in arthritis, largely because of stimulation by inflammatory cytokines such as IL-1 β . Treatment of chondrocytes with IL-1 β induces transcription of MMP-13 in vitro. IL-1β signaling converges upon the activator protein-1 transcription factors, which have been shown to be required for IL-1\beta-induced MMP-13 gene expression. Using chromatin immunoprecipitation (ChIP), we detected activator protein-1 binding within an evolutionarily conserved DNA sequence ~20 kb 5' relative to the MMP-13 transcription start site (TSS). Also using ChIP, we detected histone modifications and binding of RNA polymerase II within this conserved region, all of which are consistent with transcriptional activation. Chromosome conformation capture indicates that chromosome looping brings this region in close proximity with the MMP-13 TSS. Finally, a luciferase reporter construct driven by a component of the conserved region demonstrated an expression pattern similar to that of endogenous MMP-13. These data suggest that a conserved region at 20 kb upstream from the MMP-13 TSS includes a distal transcriptional response element of MMP-13, which contributes to MMP-13 gene expression.

Matrix metalloproteinases (MMPs)² are a family of zinc-dependent proteinases that, as a group, can break down all components of the extracellular matrix. Multiple MMPs are up-regulated in arthritis and cancer, where they are believed to be largely responsible for connective tissue remodeling (1, 2). Of

particular importance in arthritis is MMP-13, a member of the collagenase subgroup of MMPs. The degradation of type II collagen, the major type of collagen in cartilage, is considered a key step in the irreversible destruction of joint tissues. Although another collagenase, MMP-1, also degrades type II collagen, MMP-13 is the more efficient of the two (3), and thus this study focuses on the regulation of MMP-13 expression in chondrocytic cells.

In unstimulated cells, levels of MMP-13 are low (1, 4, 5). However, the transcriptional induction of MMP-13 in rheumatoid arthritis is mediated by inflammatory cytokines such as IL-1 β , which induces the expression of MMP-13 largely via the activator protein-1 (AP-1) and NF-kB family of transcription factors (4, 5). AP-1 transcription factors are well established as players in IL-1 β -induced MMP-13 expression (1). Treatment of primary chondrocytes and cell lines with IL-1 β dramatically increases transcription of MMP-13, with a concordant increase in collagen destruction (6-8). AP-1 response elements have been identified in the proximal promoter region of MMP-13, through sequence analysis and by reporter assays utilizing both stably and transiently transfected reporter constructs (5, 9, 10). A puzzling aspect of these studies, however, is that reporter constructs driven by the MMP-13 proximal promoter often do not mimic the expression pattern of endogenous MMP-13 (5, 9, 11). We speculate that perhaps this is due to the lack of other sequence elements outside of the proximal promoter, which are required for tissue-specific regulation of MMP-13 expression in chondrocytes in response to IL-1 β .

In this study, our goal was to identify novel AP-1 response elements more distal to the proximal promoter. These response elements may be necessary for appropriate regulation of MMP-13, and this may be reflected in the ability of these elements to confer an endogenous expression pattern to MMP-13 promoter-driven reporter constructs. Using ChIP, we identified Fos and Jun binding at an evolutionarily conserved region \sim 20 kb upstream from the MMP-13 transcription start site (TSS). This region also bears histone modifications consistent with those of a transcription response element. Furthermore, chromosome conformation capture (3C) data demonstrated that chromo-

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¹ To whom correspondence should be addressed: Dept. of Medicine, Dartmouth Medical School, 1 Medical Center Dr., Rubin Bldg., HB#7936, Lebanon, NH 03756. E-mail: brinckerhoff@dartmouth.edu.

² The abbreviations used are: MMP, matrix metalloproteinase; AP-1, activator protein-1; TSS, transcription start site; Pol II, polymerase II; 3C, chromosome conformation capture; qPCR, quantitative PCR.

some looping places this distal region in close proximity to the TSS. In addition, a reporter assay established this region as a response element of MMP-13 expression, and notably, expression of the response element-driven reporter construct mimicked expression of endogenous MMP-13. Thus, our data identify a novel mechanism by which IL-1 β induces MMP-13 gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—SW-1353 human chondrosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA). These cells were cultured at 37 °C with 5% CO $_2$ in DMEM (HyClone, Logan, UT) supplemented with 5% FBS (HyClone), and 2 mm glutamine. The cells were washed three times with Hanks' balanced salt solution and passaged 1:10 using 0.25% trypsin (Mediatech). Recombinant human IL-1 β (Promega, Madison WI) was solubilized in sterile H $_2$ O, stored in 10 μ g/ml aliquots at -80 °C, and added to media at a final concentration of 1 ng/ml. For experiments, SW-1353 cells were grown to \sim 90% confluence in 15-cm plates or 6-well plates and washed twice with Hanks' balanced salt solution. Two-ml (6-well plates) or 25-ml (15-cm plates) of serum-free DMEM supplemented with 0.2% lactalbumin hydrolysate, with or without IL- β was added for varying periods of time.

Chromatin Immunoprecipitation—SW-1353 cells were grown to 90% confluence in 150-mm plates ($\sim 10^7$ cells). Crosslinking was performed by adding 40 μ l of 37% formaldehyde/ml of cell culture medium directly to the culture medium, and the plates were incubated at 37 °C for 15 min. The cells were washed twice with ice-cold 1× PBS with HALT protease inhibitors (Fisher), scraped, and collected in 15-ml conical tubes on ice. The cells were pelleted by centrifugation at 2000 \times g for 5 min at 4 °C, resuspended in 1 ml of ChIP buffer (150 mm NaCl, 50 mm Tris HCl, pH 7.5, 5 mm EDTA, 0.5% Nonidet P-40, 1% Triton X-100) with protease inhibitors, and lysed on ice for 10 min. Chromatin was sonicated on ice with fifteen 15-s pulses at power setting 40 on a Sonics Vibra-Cell VC 130PB-1 ultrasonic processor. The debris was cleared by centrifugation at 12,000 \times g for 10 min at 4 °C, and the supernatant was split into 200- μ l aliquots in 1.5-ml microcentrifuge tubes for immunoprecipitation. Two µg of specific antibodies to AcH4, H3K4me2 (antibodies 06-866 and 07-030; Millipore, Temecula, CA), pan-Fos, or pan-Jun (antibodies sc-253 and sc-44; Santa Cruz Biotechnology, Santa Cruz, CA), or RNA Pol II (antibody ab5408; Abcam, Cambridge, MA) were added to each tube, and the tubes were rotated overnight at 4 °C. A 1:1 mix of protein A and protein G Dynabeads (10001D and 10003D; Invitrogen) per immunoprecipitation was washed three times and then resuspended 1:1 with ChIP buffer and distributed (40 µl/immunoprecipitation) to 1.5-ml microcentrifuge tubes. Immunoprecipitation reactions were centrifuged at 12,000 \times *g* for 10 min at 4 °C, and then 180 μl of supernatant was transferred to the protein A/G tubes and rotated for 45 min at 4 °C. The beads were collected by a magnetic rack and then washed five times by removing supernatant and resuspending in ice-cold ChIP buffer. After washing, the pellet was resuspended in 100 µl of 10% Chelex-100 (Fisher), boiled for 10 min, and then cooled on ice. One μ l of proteinase K (20 μ g/ μ l) was added to the cooled

solution, vortexed, incubated at 55 °C for 30 min, then boiled for 10 min, and centrifuged at 12,000 \times g for 1 min. Eighty μl of supernatant were transferred to a new microcentrifuge tube; 120 μl of water was added back to the original tube, vortexed, and centrifuged as before; and 120 μl of supernatant was transferred to the previous 80 μl . The samples were stored at -20 °C or immediately quantified using real time PCR with primers as indicated in Fig. 1 and Table 1, and immunoprecipitated DNA amounts were normalized to inputs and are expressed as the relative enrichment.

Chromosome Conformation Capture—We used 3C to test for looping interactions between the transcription start site and distal upstream regions. The cells were cross-linked with 1% formaldehyde, using the same procedures described in our ChIP methods (above). Digestion and ligation were performed using established procedures (12–14). Briefly, the cells were cross-linked, and then the cross-linking reaction was quenched with glycine, and cells were harvested by scraping. We then homogenized cell pellets in a Dounce homogenizer B with lysis buffer (10 mm Tris-Cl, pH 8.0, 10 mm NaCl, 0.2% (v/v) Nonidet P-40). The cell pellet was aliquoted into twenty 1.5-ml microcentrifuge tubes, $\sim 5 \times 10^6$ cells each. The cell pellets were digested in restriction enzyme buffer specific to BspHI, and the cells were incubated in 0.3% SDS. We chose BspHI because it resulted in a suitable digestion pattern at the proximal promoter and distal regions. The cells were digested with 400 units BspHI/5 \times 10⁶ cells for 36 h at 37 °C. The digested samples were then ligated with 4000 units of T4 DNA ligase for 2 h at 16 °C. The samples were then incubated overnight in proteinase K to reverse cross-links, and DNA was purified by two rounds of phenol chloroform extraction and then five rounds of ethanol precipitation. The DNA pellet was dissolved in Tris-EDTA and quantified using a NanoDrop spectrophotometer.

To control for PCR and primer efficiencies, PCR products were first made for all primer pairs spanning the region of interest (data not shown). Primers were chosen within the region from the TSS to $-25~\rm kb$ relative to the TSS, and the primers were designed such that the annealing temperatures were uniformly $58 \pm 1~\rm ^{\circ}C$. Final PCR products were analyzed using agarose gel electrophoresis.

Luciferase Reporter Assay-Luciferase reporter plasmids incorporating 1.6 kb of the MMP-13 proximal promoter (5) or 1.6 kb of the proximal promoter plus 1.5 kb flanking the ChIP-2 locus inserted immediately 5' to the promoter were constructed using the pGL3-basic plasmid (Promega). Empty pGL3-basic vector was used as a control. SW-1353 cells were plated in 6-well plates at a density of 1.5×10^5 cells/well. The next day, the cells were transiently transfected in 6-well plates with 2 µg/well of each plasmid, using 5 µl/well of Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. Four to six h after transfection, the cells were washed twice with Hanks' balanced salt solution followed by the addition of 2 ml of DMEM/lactalbumin hydrolysate medium, with or without IL-1 β for 24 h. The cells were then washed three times with cold 1× PBS, and lysates were harvested using 1× passive lysis buffer (Promega). Protein concentration was determined using Bio-Rad protein assay reagent, and equal amounts of total protein were loaded for each sample. Luciferase activity was meas-

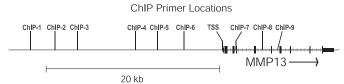


FIGURE 1. Locations of primer pairs used in ChIP experiments. We designed primers at multiple locations within the region of interest, the MMP-13 gene, and 25 kb upstream relative to the transcription start site. The primers were designed using IDT DNA PrimerQuest software with settings optimized for qPCR and confirmed to amplify a unique target using NCBI Primer-BLAST software. The specificity of each primer pair was also confirmed with melt curve analysis of gPCR amplicons and agarose gel electrophoresis (data not shown). The arrow indicates the direction of MMP-13 transcription. The black vertical bars within MMP-13 mark the location of exons, and the black blocks at the beginning and end of MMP-13 mark the 5'- and 3'-UTRs, respectively.

ured in relative light units using an Lmax II luminometer (Molecular Devices).

Statistics—All of the *p* values were calculated from triplicate samples for the difference from the IL-1 β sample as indicated in the figures, using the Student's *t* test.

RESULTS

IL-1β Induces AP-1 Transcription Factor Binding at Evolutionarily Conserved Region 20 kb Upstream from Transcription Start Site—Previous studies of MMP-13 transcriptional regulation have focused on a relatively narrow window of the MMP-13 promoter in the search for AP-1 binding sites (5, 15). In the present study, we expanded the search window to potentially identify more distal binding sites. To avoid bias, the region under examination (the transcribed region plus 25 kb upstream) was divided arbitrarily into 1-kb segments, and primers were designed for every third segment. When primers failed or we were unable to find suitable primers for a segment, we designed primers in a neighboring segment. Thus, we designed nonoverlapping primers (Fig. 1 and Table 1) tiling 25 kb upstream from the transcription start site (TSS), as well as the transcribed region of MMP-13, to scan the region for possible AP-1-binding sites.

We then conducted a time course experiment, using ChIP coupled with qPCR, with pan-Jun and pan-Fos antibodies to determine where and when AP-1 family members were binding to this 25-kb region. We added IL-1 β to the cells in serum-free medium for 30 min, 4 h, or 24 h and included untreated cells as a negative control. Cells for all conditions were cross-linked and processed simultaneously, as described above and under "Experimental Procedures." Both MMP-13 and another collagenase, MMP-1, are similarly induced by inflammatory stimuli via the AP-1 transcription factors and have a nearly identical expression profile in SW1353 cells stimulated with IL-1 β (9). For this reason, we expected the binding kinetics of Fos and Jun to be similar to those described in a previous study of AP-1 binding to the MMP-1 promoter (16). In the untreated control, constitutive levels of both Fos and Jun binding are relatively low across the region (Fig. 2). Our data show that IL-1 β treatment leads to a significant (p < 0.01) increase in Fos and Jun family members binding most prominently near the ChIP-1 primer pair. Binding was detected at 30 min and 4 h after treatment with IL-1 β and returned to control levels by 24 h. There was

also an increase in Fos binding at the ChIP-6 locus at 30 min and 4 h, and an increase in the Jun-binding region-wide at the 4-h time point. However, there was no definitive increase in Jun binding at ChIP-6, and because the two bind to form the AP-1 heterodimeric transcription factor, this suggests that binding of Fos at the ChIP-6 site may not be functional. Furthermore, detection of both Fos and Jun serves as an internal control for the experiment, and both are involved in the IL-1 β induction of MMP-13 expression (17).

DNA sequence alignment data from the University of California, Santa Cruz genome browser, shows that ChIP-1, -2, and -3 are within a region that contains conserved sequence among vertebrates (Fig. 3A) (18) and that this region contains several consensus AP-1 sites (Fig. 3B), suggesting a biologically relevant role (see below). The ChIP-6 locus does not appear to be conserved (data not shown). Although this does not rule out a potential role for this site in the regulation of MMP-13 gene expression, we chose to focus instead on the more distal region.

ChIP-2 and ChIP-3 Loci Are Enriched for AcH4 and H3K4me2—The sequence conservation and the binding of AP-1 transcription factors determined by ChIP (Fig. 2) led us to consider the presence of a functional element near the ChIP-1 locus. Therefore, we sought to determine whether this region bore other hallmarks of a transcriptional response element, such as the presence of the histone modifications, acetylated histone H4 (AcH4) and dimethylated lysine 4 of histone H3 (H3K4me2), which are indicative of both actively transcribed regions and transcriptional response elements (19).

Using the same approach as in our previous AP-1 ChIP, we scanned the transcribed and upstream regions of MMP-13 for H3K4me2- and AcH4-enriched sites. The cells were left untreated or treated with IL-1 β for 24 h and then analyzed by ChIP for enrichment of H3K4me2 and AcH4.

The AcH4 modification was induced by IL-1 β throughout the entire region, but a clear increase can be seen within the transcribed region (Fig. 4A, ChIP loci 7-9), as expected for an actively transcribed gene. Importantly, however, a significant (p < 0.01) increase in AcH4 levels was also detected at the ChIP-2 and ChIP-3 loci (Fig. 4A), in agreement with our hypothesis that this region contains an active IL1- β response element (see below). The transcribed region was also enriched for the H3K4me2 modification (Fig. 4B), consistent with the histone modification profile of a transcriptionally active gene. In addition, the levels of H3K4me2 were significantly (< 0.01) elevated at ChIP-2, with or without stimulation with IL-1 β , in agreement with previous observations associating an increase in H3K4me2 with transcriptional response elements (19). We confirmed and extended these findings by delineating AcH4 and H3K4me2 marks in a time course experiment (supplemental Fig. S1). The data agree with those presented in Fig. 4 and indicate that these regions are engaged early in the transcriptional activation of MMP-13, coincident with the recruitment of Fos and Jun binding.

However, neither the AcH4 nor the H3K4me2 modifications were substantially enriched at ChIP-1. The lack of AcH4 and H3K4me2 at ChIP-1 does not rule out this locus as a component of the response element, because the resolution of these ChIP experiments is such that the precise AP-1 binding site(s)



TABLE 1

Primers	Forward primer $(5' \rightarrow 3')$	Reverse primer $(5' \rightarrow 3')$
ChIP Primers		
ChIP1	GGGCTGTGAAGCAGCTGAGTATTT	TGGCCAGGATGATGTTACAGTGGT
ChIP2	AGGGCATGACTCACAAACTCTCCT	GTCATGTTCTCTGGCTTCAACCCT
ChIP3	GTGCCCTGTGCAGTTCAAAGACAT	AAGAAATGGGTCCCAGAAACCGCT
ChIP4	CCCAAAGTCGGGCAAATGTGGAAT	TTGATTCAGCAAGCCATCAGGCAG
ChIP5	CCTGTTCCTTTGTGCTGTGCTCAT	TGAAGGAGAAAGGTCCAGCCA
ChIP6	ATACGACACCCGAAGCAAGTCACA	TTGAGGGCAGCATCTCAGTCTTCA
TSS	GTCGCCACGTAAGCATGTTTACCT	CTGTTGTCTTTCCGCAGAGATTACC
ChIP7	AGAGCATGGCGACTTCTACCCATT	TCTCCTCCATAATTTGGCCCAGGA
ChIP8	AGGTTGGTGAAAGTGAGATCC	TACCACAGGCCACTTGAGAGGTTT
ChIP9	AGGCCATGAGACCATGGAAGACAT	GCAAATGTGCCAGGCATTGTGTTG
3C Primers	Primer $(5' \rightarrow 3')$	
Anchor	CCTGATATGACTCATTCTGAAGTCGAAAAGGC	
1	GACTTCAGACCATATTTCAAGGAAGGATGGG	
2	GGCAGATGAGGTAGAAATCAGTGAACTGG	
3	GGGCTGTGAAGCAGCTGAGTATTT	
4	GGAAGTGAGACCACAGCAGTCTAGATTTAC	

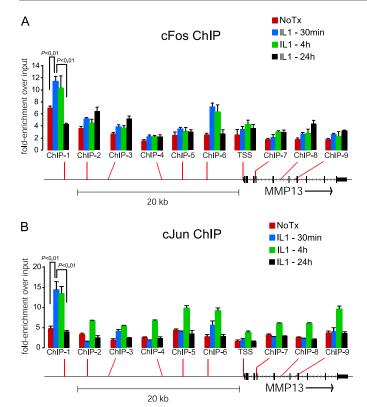


FIGURE 2. c-Fos and c-Jun ChIP results in SW1353 cells stimulated with **IL-1β.** Chromatin for ChIP assays was isolated from SW1353 chondrosarcoma cells that were treated with IL-1 β at 1 ng/ml in serum-free medium for 30 min, 4 h, or 24 h; untreated cells were used as a control. Chromatin was cross-linked with 1% formaldehyde and then sonicated to an average size of 500 bp. Sonicated chromatin was incubated with antibodies to either pan-Fos or pan-Jun, and antibody-bound chromatin was immunoprecipitated with protein A- and protein G-conjugated magnetic beads. Immunoprecipitated chromatin was purified and used as the template in qPCR using primers indicated in Fig. 1. The data indicate fold enrichment of the qPCR signal of immunoprecipitated DNA (normalized to total immunoprecipitated DNA as quantified by PicoGreen quantification; Invitrogen) relative to the qPCR signal of input DNA (normalized to amount of input DNA as quantified by PicoGreen). The results are presented as the means and standard deviations of independent experimental triplicates and qPCR duplicates. NoTx, no treatment. p < 0.01 for no treatment versus IL-1 β at 30 min; and for IL-1 β at 4 h versus 24 h. Also, p < 0.05 for ChIP 1 locus at 30 min versus all other loci at 30 min (not shown for simplicity).

and boundaries of the AcH4/H3K4me2-enriched region cannot be determined. In agreement with our AcH4 and H3K4me2 ChIP results, data from the University of California, Santa Cruz genome browser and the ENCODE Project indicate the pres-

ence of Fos/Jun binding site(s) as determined by ChIP-seq within the ChIP-2 locus (Fig. 3*B*) (20). The University of California, Santa Cruz/ENCODE Project data do not coincide precisely with our Fos/Jun ChIP findings, as shown in Fig. 2; perhaps this discrepancy is due to differences in the resolution with our ChIP methodology compared with the ChIP-seq method used to generate the Fos/Jun site from the ENCODE Project (see "Discussion" below) (20).

Nonetheless, our data highlight a region that is involved in transcriptional regulation of MMP-13 and that contains several AP-1 consensus sites (Fig. 3*B*). Regulatory regions such as response elements are often conserved (21), and sequence analysis indicates that the ChIP-2 and ChIP-3 loci (along with ChIP-1 as previously mentioned), are conserved among vertebrates (Fig. 3*A*). Thus, the AcH4/H3K4me2 ChIP data suggest, along with the previous AP-1 ChIP data and sequence conservation, that the region around ChIP-1–3 is important for the regulation of MMP-13 expression.

Chromosome Conformation Capture Demonstrates a Direct Interaction between TSS and Distal Response Element—The data presented thus far support a role for the ChIP-1–3 region in regulating transcription of MMP-13. However, if direct interaction between response element-bound transcription-regulating factors and the transcription start site is required for regulating transcription, the large distance between this region and the transcription start site must be overcome. This would necessitate chromatin looping in order for regulatory factors bound at the ChIP-1–3 loci to have a direct effect upon the transcription start site (22). To test for potential interactions between the MMP-13 transcription start site and more distal elements, we performed 3C capture as previously described (see "Experimental Procedures").

The cells were left untreated as a control or treated with 1 ng/ml IL-1 β for 24 h and then treated with formaldehyde to form cross-links between any interacting DNA regions. The cross-linked chromatin was digested with the restriction endonuclease BspHI, and the digested DNA was treated with DNA ligase. Interactions between DNA elements would then be detectable with PCR utilizing primers designed to detect novel strands of DNA formed from the ligation of the cross-linked and digested products. See Fig. 5 for the location of 3C primers relative to the location of the ChIP primers.

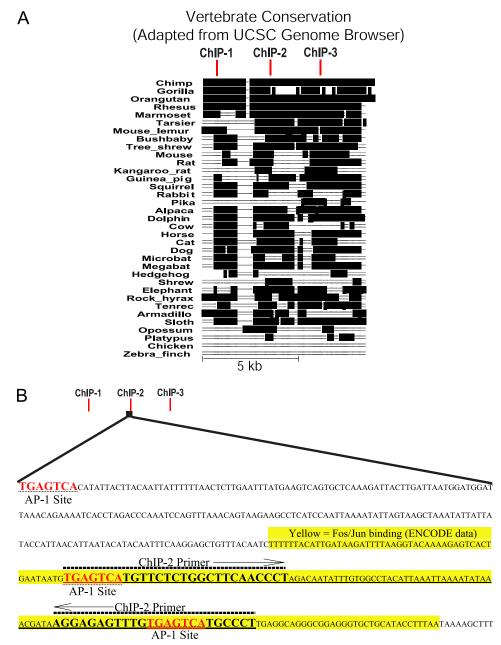
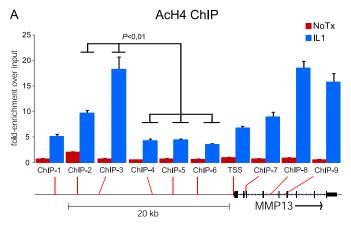


FIGURE 3. Vertebrate sequence alignment illustrating conserved sequence clusters. A, image generated from the University of California, Santa Cruz Genome Browser, Vertebrate Multiz Alignment & Conservation (see website for a description of the "conservation track" methods). The graph illustrates the alignment of human DNA sequence with 35 vertebrates in a region centered around 20 kb upstream from the MMP-13 TSS. The black blocks indicate regions of relatively high conservation. Relevant ChIP primer locations are marked at the top of the figure (red vertical lines). B, DNA sequence for part of ChIP-2 locus, showing documented Fos/Jun binding site and additional conserved AP-1 sites within this region, as derived from the ENCODE Project (20).

We found that in untreated cells, there is little interaction detected between the MMP-13 TSS and the sites tested, except for a weak interaction at primer 2 (Fig. 6, A and B). Fig. 6C is a positive control, which shows that all four primers displayed relatively equal efficiency. However, in IL-1 β -treated cells, there is a marked interaction at primer 2. This indicates that DNA at or near the primer 2 locus is interacting directly with the MMP-13 TSS and/or proximal promoter region. Our data indicate that this interaction may be inducible, or at least strengthened, by treatment with IL-1β. Perhaps most importantly, the 3C primer 2 is in the ChIP-1–3 region, 5' relative to ChIP-2, which we have already demonstrated to be a likely can-

didate response element, with evolutionarily conserved DNA sequence, AP-1 transcription factor binding, and histone modifications consistent with a transcriptional response element.

RNA Pol II Binds to ChIP 2 Locus, Consistent with Transcriptional Activation in This Region—To support the 3C data, we performed a ChIP for Pol II binding with an IL-1 β time course (Fig. 7). We reasoned that if the distal DNA interactions we identified with 3C were functional, we should detect Pol II binding at this distal element. Indeed, even in untreated cells, we found significant (<0.05) Pol II binding within the ChIP 2 region, suggesting that Pol II may be poised for transcriptional activation at this locus. These findings further demonstrate the



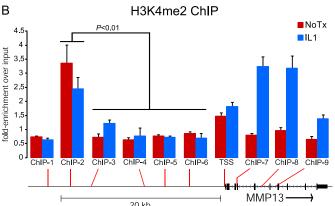
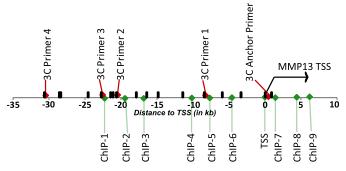


FIGURE 4. AcH4 and H3K4me2 ChIP results in SW1353 cells stimulated with IL-1β. Chromatin for ChIP assays was isolated from SW1353 chondrosarcoma cells that were treated with IL-1 β at 1 ng/ml in serum-free medium for 24 h; untreated cells were used as a control. Chromatin was cross-linked with 1% formaldehyde and then sonicated to an average size of 500 bp. Sonicated chromatin was incubated with antibodies to either AcH4 (A) or H3K4me2 (B), and antibody-bound chromatin was immunoprecipitated with protein A- and protein G-conjugated magnetic beads. Immunoprecipitated chromatin was purified and used as the template in qPCR using primers indicated in Fig. 1. The data indicate fold enrichment of the qPCR signal of immunoprecipitated DNA (normalized to total immunoprecipitated DNA as quantified by PicoGreen quantification; Invitrogen) relative to the qPCR signal of input DNA (normalized to amount of input DNA as quantified by PicoGreen). The results are presented as the mean and standard deviation of independent experimental triplicates and qPCR duplicates. For A, p < 0.01 for IL-1 $\dot{\beta}$ -treated ChIP-3 locus versus IL-1 β -treated ChIP-4, -5, and -6 loci). For B, p < 0.01 for ChIP-2 locus, untreated, and IL-1 β -treated versus ChIP-4, -5, and -6 loci.



■ - BspHI sites

FIGURE 5. Locations of primers used in 3C experiment, relative to the location of ChIP primers. 3C primers were designed as described under "Experimental Procedures" and are shown here (top, loci marked in red), relative to the location of the ChIP primers (bottom, loci marked in green). The 3C anchor primer was immediately 3' relative to the MMP-13 TSS.

role of the ChIP 2 region in mediating transcriptional activation in response to an inducer, such as IL-1 β .

Importantly, the data support the activation of the regions identified in Figs. 2 and 4. In addition, the time frame of activation agrees with our previous findings, which measured a time course of MMP-13 heterogeneous nuclear RNA (hnRNA) induction in these same cells in response to IL-1 β (4). It is noteworthy that the proximal promoter (TSS) and the transcribed regions (ChIP-7, -8, and -9) also show increased binding of Pol II, serving as a positive control and substantiating a role for this upstream region in MMP-13 transcription.

Luciferase Reporter Construct Driven by 1 kb of Sequence Flanking ChIP-2 Is IL-1 β -inducible and Mimics Expression Pattern of Endogenous MMP-13—Previous studies used a luciferase reporter system to test the ability of the MMP-13 proximal promoter to drive expression (5, 11). Neither 1.6 kb of the proximal promoter nor repeats of the proximal AP-1-binding site were able to drive reporter expression in a manner that mimicked the expression pattern of endogenous MMP-13.

We posited that these reporter constructs failed because the DNA was not in its native chromatin environment and thus lacked other regulatory DNA elements. Considering the sequence conservation and ChIP data at the putative distal response element identified above, we tested whether this element was one of the missing regulatory elements that may restore a more endogenous-like expression pattern to the reporter construct.

We cloned 1.5 kb of DNA in the region surrounding ChIP-2 and inserted it 5' relative to 1.6 kb of the MMP-13 proximal promoter in the pGL3-basic luciferase reporter plasmid. We chose the ChIP-2 segment to test in our reporter assay because it gave the most response element-specific signal in our ChIP assays. Similar to previous studies (5, 9), the proximal promoter construct was not regulated by IL-1 β . However, we found that the ChIP-2-containing reporter construct exhibited an expression pattern that closely resembled expression of endogenous MMP-13 (Fig. 8). Specifically, luciferase activity was induced by treatment with IL-1 β , correlating with endogenous MMP-13 mRNA and protein (11). A construct in which this distal region was inserted in the reverse orientation was not as effective in mediating transcriptional induction by IL-1 β (data not shown), thereby suggesting that this element may not function as a bone fide enhancer. Furthermore, an analogous construct containing the ChIP-1 region failed to transactivate gene expression (data not shown). Therefore, we conclude that, within the 1.5 kb of DNA flanking the ChIP-2 region, there exists a DNA element or elements that confer an appropriate response to IL-1 β in conjunction with the MMP-13-proximal promoter region.

DISCUSSION

Using ChIP, we identified a distal transcriptional response element of MMP-13, an evolutionarily conserved region \sim 20 kb upstream from the MMP-13 TSS. The response element is identified by two characteristic histone modifications, H3K4me2 and AcH4, as well as by increased binding of RNA polymerase II. As demonstrated by our Fos and Jun ChIP data, the conserved region also contains functional binding sites for AP-1 transcription factors, which are required for induction of



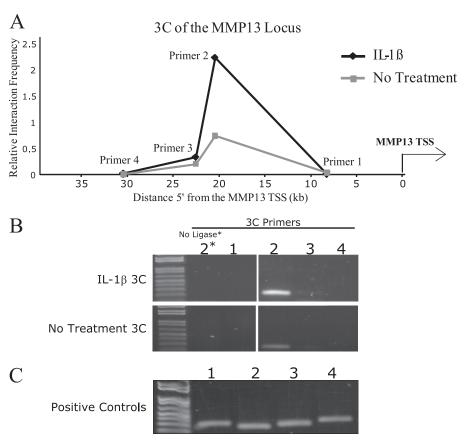


FIGURE 6. Chromosome conformation capture detects interaction between distal response element and the transcription start site. Chromosome conformation capture of the MMP-13 locus with IL-1 β stimulation. Chromatin for 3C analysis was isolated from SW1353 chondrosarcoma cells that were treated with IL-1 β at 1 ng/ml in serum-free medium for 24 h; untreated cells were used as a control. Chromatin was fixed with 1% formaldehyde as described in the ChIP methods. Cell pellets were digested with 400 units BspHI/5 imes 10 6 cells, and the digested samples were then ligated with 4000 units of T4 DNA ligase. DNA was purified by phenol chloroform extraction and ethanol precipitation. The resultant samples were used as the template in PCR with primers designed within the region from the TSS to -25 kb relative to the TSS. The anchor primer is near the TSS, and target primers are on the distal (5') side of BspHI sites within the region described above. A, graph indicates quantified 3C results for primers at selected BspHI sites spanning 50 kb 5' of the MMP-13 gene. Interaction frequency is relative to positive control amplifications. B, gel images of the 3C results in the presence and absence of IL-1B. No ligase lanes for primer 2 control for nonspecific amplification. C, positive control PCR bands for primers 1-4 in combination with the MMP-13 promoter, indicating relatively equal primer efficiency.

MMP-13 transcription by IL-1β. 3C data document a direct interaction between this distal region and the TSS, enabling factors bound at the response element to directly influence transcription of MMP-13. Lastly, a luciferase reporter assay clearly supports the hypothesis that the region acts as a distal response element for MMP-13 transcription. Notably, expression of the response element-driven reporter construct correlates with expression of the endogenous MMP-13 gene, showing induction with IL-1 β treatment. This finding contrasts sharply with our previous studies, in which constructs harboring only the proximal failed to show induction by IL-1 β (5, 11).

The ChIP data demonstrate Fos and Jun binding at the ChIP-1 locus, ∼20 kb upstream of the MMP-13 promoter. To be considered as functional with respect to MMP-13 regulation, this binding should ideally be inducible with IL-1 β treatment, because IL-1 β ultimately drives expression of MMP-13 via the AP-1 transcription factors. Indeed, Fos and Jun binding is inducible in cells treated with IL-1 β . It is important to note that both Fos and Jun were detected at the response element, because the two factors are required for IL-1 β induction of MMP-13, and Fos and Jun bind to each other to form the AP-1 heterodimer; if the intact heterodimer is binding to the

response element, one would expect to detect both AP-1 family members. Both MMP-1 and MMP-13 require AP-1 to be induced by IL-1 β , and both genes show nearly identical expression patterns in SW1353 cells treated with IL-1 β (4, 9). The AP-1 ChIP data are temporally consistent with a previous study by Martens et al. (16), in which they describe the Fos and Jun binding kinetics at AP-1 response elements in the MMP-1 promoter.

The large distance separating the AP-1-bound region from the transcription start site predicates that if this region is regulating MMP-13 transcription, then it is doing so as a distal response element. Acetylated histone H4 and dimethyl-H3K4 are both histone modifications that are associated with transcriptional response elements (19, 23), and our ChIP data clearly show enrichment for these marks in the ChIP-1-3 region (Fig. 4), as would be expected at a transcriptional response element. Furthermore, our time course study (supplemental Fig. S1) indicates that this enrichment occurs along with the recruitment of Fos and Jun to this upstream region. Importantly, our findings also demonstrate the presence of RNA Pol II binding within this region (Fig. 7), again indicating transcriptionally active DNA. Finally, a hallmark of a functionally impor-

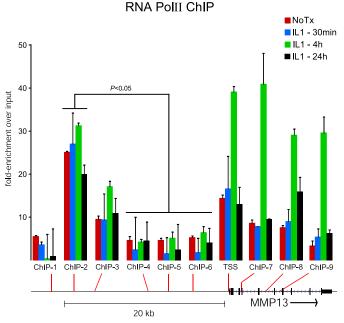


FIGURE 7. RNA Pol II ChIP results in SW1353 cells stimulated with IL-1 β . Chromatin for ChIP assays was isolated from SW1353 chondrosarcoma cells that were treated with $IL-1\beta$ at 1 ng/ml in serum-free medium for 30 min, 4 h, or 24 h; untreated cells were used as a control. Chromatin was cross-linked with 1% formaldehyde and then sonicated to an average size of 500 bp. Sonicated chromatin was incubated with antibody to Pol II, and antibody-bound chromatin was immunoprecipitated with protein A- and protein G-conjugated magnetic beads. Immunoprecipitated chromatin was purified and used as the template in qPCR using primers indicated in Fig. 1. The data indicate fold enrichment of the qPCR signal of immunoprecipitated DNA (normalized to total immunoprecipitated DNA as quantified by PicoGreen quantification; Invitrogen) relative to the qPCR signal of input DNA (normalized to amount of input DNA as quantified by PicoGreen). The results are presented as the means and standard deviations of independent experimental triplicates and qPCR duplicates. p < 0.05 for ChIP-2 locus versus ChIP-4, -5, and -6 loci. NoTx, no treatment.

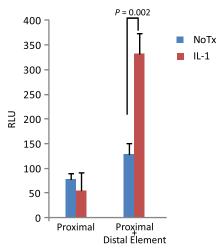


FIGURE 8. A luciferase reporter driven by the MMP-13 proximal promoter combined with the distal response element is responsive to IL-1 β and mimics the response of endogenous MMP-13. SW-1353 cells were seeded in triplicate in 6-well plates and transfected with 2 μ g/well of the 1.6-kb luciferase; response element 1.6-kb luciferase; or the empty vector pGL3-basic (see "Experimental Procedures") luciferase reporter constructs. The cells were then treated for 24 h with 1 ng/ml IL-1 β for 24 h; untreated cells were used as a control. The cells were solubilized in passive lysis buffer, and equal amounts of protein were loaded for each sample and assayed for luciferase activity as reported in relative light units (RLU). The error bars represent standard deviations of biological triplicates. p=0.002 for response element 1.6-kb luciferase treated with IL-1 β versus no treatment (NoTx).

tant segment of DNA is sequence conservation through evolution, and the sequence at ChIP-1–3 is highly conserved among vertebrates (Fig. 3*A*).

The fact that Fos/Jun binding to the ChIP-1 locus does not coincide precisely with the binding of histone markers and Pol II does not rule out this locus as a real component of this response region. However, sequence analysis does not show an obvious AP-1-binding site at ChIP-1, and data from the ENCODE Project document AP-1 binding within the ChIP-2 locus, which contains at least two consensus AP-1 response elements (Fig. 3B) (20), thereby suggesting that ChIP-2 may be the functional locus. Thus, the binding of Fos and Jun to ChIP 1 (Fig. 2) may reflect the limits of resolution of our methodology versus the more detailed technique of ChIP-seq (20). Alternatively, it is possible that Fos and Jun binding is simply offset by physical constraints imposed by the co-localization of other proteins involved in transcriptional activation (Figs. 4 and 7 and supplemental Fig. S1). Nonetheless, our data delineate a distal upstream region of the MMP-13 promoter DNA that is functionally involved in the transcriptional regulation of this gene.

Our 3C data point out a long range interaction between the putative response element and the TSS. The interaction between the TSS and this region would potentially bring the distant AP-1 transcription factors into close proximity with the general transcription machinery at the TSS. This would enable the distal response element and its associated transcription factors and co-regulatory proteins to directly regulate transcriptional activity at the MMP-13 TSS, thus elucidating a general mechanism of MMP-13 transcriptional regulation. A characteristic of response elements is their ability to drive expression in reporter assays, and our data clearly show the ability of the ChIP-2 locus to drive expression of a luciferase reporter, when cloned 5' relative to 1.6 kb of the proximal promoter region. Furthermore, the transiently transfected reporter construct driven by components of the MMP-13 promoter and distal response element mimicked expression of endogenous MMP-13 in cells treated with IL-1 β . The results in the present study are in keeping with the current paradigm of transcriptional regulation, which has moved beyond the more simplistic mechanism of proximal promoter-regulated transcription to include distal regulatory elements that are required for proper transcriptional control. We conclude that our data designate an upstream region as a distal IL-1 β response element for MMP-13.

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